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During August and September 1988, we collected adult mosquitoes from 14 locations in the Senegal River basin to search for evidence of Rift Valley fever (RVF) viral activity one year after the 1987 outbreak, which occurred along the Senegal-Mauritania border.More than 62,000 specimens representing 18 species in seven genera were collected with carbon dioxide-baited, solid-state Army miniature light traps and sheep-baited traps. Twenty virus isolations from Culex,Aedes, and Anopheles mosquitoes were recovered from six locations: Fanaye Diery(11),Bode(four),Matam(two),Diongui (one),Ndialene(one), and Ngoui(one).Species yielding viral isolates were Anopheles pharoensis (eight).Culex triaeniorhynchus(three).Cx.univitatus gr.(three),Cx.antennatus (two),Cx.poicilipes(two),Ae.hirsutus(one),and An.gambiae(one). Viruses were identified by complement fixation, and virus and plaque-reduction neutralization testing as Ngari(Bunyavirus, Bunyaviridae) (n=15),Babanki(Alphavirus,Togaviridae)(n=3).Bagaza (Flavivirus,Flaviviridae)(n=1),and Bangui(Bunyavirus-like)(n=1).No evidence of any RVF viral activity in the Senegal River Basin was detected in the mosquitoes tested.

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ARBOVIRUS ISOLATIONS FROM MOSQUITOES COLLECTED DURING 1988 IN THE SENEGAL RIVER BASIN

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Abstract. During August and September 1988, we collected adult mosquitoes from 14 locations in the Senegal River basin to search for evidence of Rift Valley fever (RVF) viral activity one year after the 1987 outbreak, which occurred along the Senegal-Mauritania border. More than 62,000 specimens representing 18 species in seven genera were collected with carbon dioxide–baited, solid-state Army miniature light traps and sheep-baited traps. Twenty virus isolations from *Culex*, *Aedes*, and *Anopheles* mosquitoes were recovered from six locations: Fanaye Diery (11), Bode (four), Matam (two), Diongui (one), Ndialene (one), and Ngoui (one). Species yielding viral isolates were *Anopheles pharoensis* (eight), *Culex tritaeniorhynchus* (three), *Cx. univitattus* gr. (three), *Cx. antennatus* (two), *Cx. poicillipes* (two), *Ae. hirsutus* (one), and *An. gambiae* (one). Viruses were identified by complement fixation, and virus and plaque-reduction neutralization testing as Ngari (*Bunyavirus*, Bunyaviridae) (n = 15), Babanki (*Alphavirus*, Togaviridae) (n = 3), Bagaza (*Flavivirus*, Flaviviridae) (n = 1), and Bangui (Bunyavirus-like) (n = 1). No evidence of any RVF viral activity in the Senegal River Basin was detected in the mosquitoes tested.

A major epizootic of Rift Valley fever (RVF) occurred in October–December 1987 in the Senegal River basin, centered around the town of Rosso on the Senegal-Mauritania border (Figure 1).[1] Cases of human febrile illness and often fatal hemorrhagic disease were first noted by French physicians at the Rosso hospital. In all, 385 laboratory-confirmed human infections of RVF were reported by the Institut Pasteur in Dakar, Senegal, and estimates of total human involvement exceeded 1,000 cases.[2,3] Local domestic animals, primarily sheep and goats, were also heavily involved in the outbreak. Serosurveys of domestic animals in the Rosso area revealed IgM antibody to RVF virus in 80% of the domestic animals tested.[4]

The magnitude of this outbreak and its apparent linkage to the development of a multinational irrigation project[5] generated intense interest in conducting studies during the rainy season of 1988 (August–September) to assess the potential for continuing RVF viral activity in the Senegal River basin. This study describes attempts to isolate virus from mosquitoes collected in the Senegal River basin during that rainy season.

MATERIALS AND METHODS

Study area

Mosquitoes tested in this study were collected at 14 locations in the Senegal River Basin (Figure 1). The vegetative pattern in the Senegal River basin can be categorized as Sahelian to Sudano-Sahelian savannah, dominated by annual grasses and widely dispersed trees, particularly *Acacia* spp. and *Combretacae* spp.[6] Rainfall occurs primarily from July through October and ranges from 200 mm to 600 mm annually. A water development program, instituted by the governments of Senegal, Mauritania, and Mali, was recently established in this area to make better use of the Senegal River basin for agricultural purposes. Two dams were constructed on the Senegal River, one at Diama in the delta region, preventing saltwater incursion upstream, and a second at Manantali in Mali, creating a reservoir

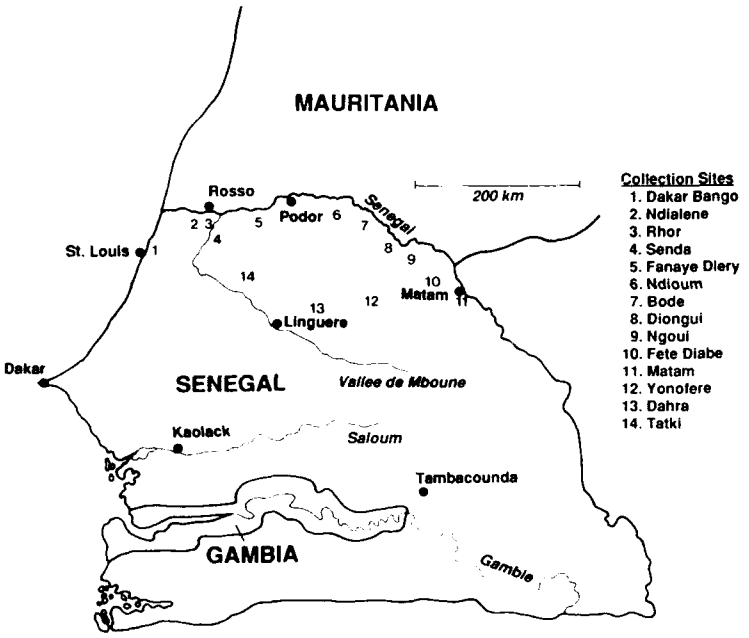

FIGURE 1. Map of the 14 mosquito collection sites at villages in Senegal. Rivers are indicated by italicized letters.

to maintain a constant level in the river and encourage the development of irrigation agriculture in the region.[7]

Mosquito collection and identification

Mosquitoes were sampled from 14 villages during August and September 1988 in sheep-baited traps and in solid-state Army miniature light traps (John W. Hock, Co., Gainesville, FL) baited with 0.5 kg of dry ice. Collections were frozen in liquid nitrogen in the field and stored at −70°C upon return to the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID). Mosquitoes were identified to species by using available identification keys,[8–10] sorted to sex and blood-feeding status, and pooled on a refrigerated (4°C) table. Specimens were stored at −70°C until processed for virus isolation. Identifications of specimens used as vouchers for specimens that were assayed were verified by the Walter Reed Biosystematics Unit, Smithsonian Institution (Washington, DC).

Virus isolation

Mosquito pools were triturated in glass tissue grinders containing 3.0 ml of diluent as described by Turell and Bailey.[11] The homogenized suspensions were centrifuged at 800 × *g* for 20 min and the supernatant was immediately inoculated onto two plates of Vero cell monolayers (grown in 12-well plastic plates). After adsorption for 1 hr at 35°C, the cells were overlayed with 2 ml/well of an agarose-nutrient suspension containing 0.5% (w/v) agarose in Eagle's basal medium with Earle's salts (plate 1) and a liquid maintenance medium consisting of medium 199 with Earle's salts, supplemented with 10% fetal bovine serum (plate 2). Cell cultures were then incubated at 35°C in a humidified atmosphere of 5% CO_2. Liquid cultures were observed daily for cellular cytopathic effects (CPE) for 6–7 days, while agarose-overlayed cells were stained on day 5 by applying a second agarose overlay (1 ml/well) containing neutral red (330 μg/ml). Agarose-overlayed cells were incubated at 35°C for

one additional day, and were then observed for the presence of plaques.

Preparation of seed stocks

Cell culture seed stocks were prepared for each positive mosquito pool by inoculation (0.2 ml undiluted) of either a plaque-picked suspension or cell culture supernatant from the original isolation assay onto fresh Vero cell monolayers grown in 25-cm^2 (T-25) plastic flasks. Viral titers for each seed stock were determined by plaque assay in Vero cells, and expressed as $\log_{10}$ plaque-forming units (PFU) per ml. Isolates were identified at the laboratories of both the USAMRIID and the Centre Collaborateur Organization Mondial del la Sud de Reference et de Recherche pour les Arbovirus, Dakar, Senegal.

Preparation of immune sera

Mouse immune ascitic fluids were prepared for a selected number of the viral strains isolated by using a modification of the method described by Brandt and others.[12] A 1:100 dilution of each cell culture seed stock was inoculated intracerebrally into litters of 1–3-day-old suckling mice (ICR strain; 0.03 ml/mouse) to provide infected mouse brains to immunize a group of mice for production of immune ascitic fluid. After the ascitic fluid was harvested, it was treated to eliminate clot formation, as described by Chiewsilp and McCown.[13] Five milliliter aliquots of immune serum were stored at −20°C. Normal mouse brain extract was used to prepare normal ascitic fluid.

Complement fixation (CF) test

Preparation of antigens. Crude complement-fixing antigens were prepared for each viral isolate as 10% (w/v) infected suckling mouse brain suspensions in Tris-buffered saline, pH 9.0 or infected Vero cell culture supernatant fluid.[14] Normal mouse brain and uninfected Vero cell supernatant control antigens were prepared similarly.

The CF test was used to rapidly screen and tentatively identify the viral isolates. Serial four-fold or ten-fold dilutions (starting at 1:4) of antigens were tested against various dilutions (two-fold starting at 1:8) of homologous hyperimmune sera and a battery of type-specific heterologous hyperimmune sera in a cross-block titration performed in 96-well microtiter plates.[14] A complement-control titration was included in each assay to detect any anticomplementary activity in the immune sera or the viral antigen preparations.

Plaque reduction and virus neutralization tests in mice

The plaque-reduction neutralization test (PRNT) was performed in the same manner as the plaque assay for infectious virus, except that a known concentration of virus (50–100 PFU) of each viral strain was incubated at 37°C for 30 min with various dilutions of either National Institutes of Health (NIH; Bethesda, MD) arbovirus reference reagents or type-specific immune sera before inoculation onto Vero cell monolayers.[15] An immune serum was considered to react positively with a viral strain when it inhibited ≥ 50% of the virus dose. When antisera to some viruses were not available at the USAMRIID, virus neutralization tests in mice, instead of PRNT, were conducted at the Institute Pasteur in Dakar, Senegal. Virus neutralization tests were performed in three-day-old mice inoculated intracerebrally with 0.02 ml of an incubated mixture of test serum (1:4 dilution) and virus (serial 10-fold dilutions) according to the methods of Causey and others.[16]

RESULTS

A total of 62,055 mosquitoes representing 18 species and seven genera was collected. *Culex* (eight species, 92.7%), *Anopheles* (four species, 3.8%), and *Aedes* (four species, 2.6%) accounted for more than 99% of all specimens. Twenty viral isolates were recovered from 2,793 pools of unfed female mosquitoes (Table 1). All 20 isolates produced visible plaques in Vero cells by day 6 postinoculation, while 19 of 20 strains exhibited viral CPE in liquid-overlayed cells. Passage of either infected cell culture supernatant or plaque-picked material of each isolate resulted in characteristic viral CPE within 4–7 days postinoculation. Attempts to reisolate virus from the original mosquito pools were successful in both cell culture systems used.

Virus identification

Plaque-reduction neutralization test. Preliminary screening of the viral isolates was performed

TABLE 1
Mosquitoes tested for virus isolation

Species	No. tested	Virus isolations*	MFIR†
Aedes			
hirsutus	1,046	1 Ngari	1:1,046
irritans	24	0	
ochraceus	88	0	
sudanensis	480	0	
spp.	3	0	
Aedomyia africanus	26	0	
Anopheles			
gambiae complex	316	1 Ngari	1:316
pharoensis	1,906	7 Ngari	1:272
		1 Bangui	1:1,906
rufipes	66	0	
ziemanni	89	0	
spp.	10	0	
Culex			
antennatus	21,209	2 Ngari	1:10,604
bitaeniorhynchus	78	0	
perfuscus	347	0	
poicilipes	5,567	2 Ngari	1:2,783
quinquefasciatus	73	0	
thalassius	1,097	0	
tritaeniorhynchus	20,081	2 Ngari	1:10,040
		1 Babanki	1:20,081
univittatus group	5,565	2 Babanki	1:2,782
		1 Bagaza	1:5,565
spp.	3,568	0	
Mansonia uniformis	381	0	
Mimomyia spp.	1	0	
Uranotaenia spp.	34	0	
Total	62,055	20	

* All virus isolations were made from pools of unfed females.
† MFIR = minimum field infection rate (no. of virus isolates/no. of mosquitoes tested).

by PRNT in Vero cells using NIH arbovirus reference grouping sera and type-specific immune mouse sera. Three isolates reacted positively with group A reference serum and with anti-Sindbis (SIN) mouse ascitic fluid. 15 isolates were markedly inhibited by the Bunyamwera (BUN) serogroup reference serum, and two viral strains (16-17 and 20-100) showed no reactivity with any of the immune sera used. Control viruses showed positive reactivity with the appropriate antisera.

Complement fixation test. Based on results obtained from preliminary neutralization tests and characteristic growth patterns in cell culture and suckling mice, the viral isolates were categorized into different groups for screening by the CF test. Group 1 consisted of three isolates (12-401, 20-130, and 22-17) that reacted with group A reference antiserum and with SIN ascitic fluid. The 15 isolates that reacted with BUN group reference serum and showed similar patterns of growth in Vero cell culture and suckling mice were placed in group 2. The remaining two viral strains (16-17 and 20-100) that showed no reactivity in PRNTs, but had similar growth characteristics, were placed in group 3.

When assayed by the CF test, isolates in group 1 showed antigenic similarity to one another and to SIN virus. In contrast, results obtained with other alphavirus antigens and immune sera (Chikungunya, Semliki Forest, Ndumu, and Middelburg) showed virtually no reactivity when tested with group 1 viral strains. However, preliminary results with neutralization tests of these viruses in mice showed close antigenic similarity to Babanki (BBK) virus.

All group 2 CF antigens reacted positively and similarly when tested with immune serum pro-

TABLE 2

Serologic identification of isolate 20-100 by the plaque-reduction neutralization test

Viral strain†	Hyperimmune mouse ascitic fluid*	
	20-100	Bagaza
20-100	>640	40
Bagaza	>640	640

* Values are the reciprocal dilution inhibiting 80% of a plaque dose of approximately 50–100 plaque-forming units as assayed on Vero cell monolayers.
† Virus was prepared from infected Vero cell supernatants.

TABLE 3

Serologic identification of isolate 16-17 by virus neutralization tests in mice

Antisera	Antigen*	
	16-17 (6.1)†	Bangui (6.6)†
16-17	4.4‡	3.3
Bangui	2.8	5.1

* Antigens were prepared from mouse brain.
† Values are the $\log_{10}$ titer of antigen with normal mouse serum.
‡ Neutralization index ($\log_{10}$ plaque-forming units of virus neutralized with type-specific antiserum).

duced against one of the isolates (14-144). All isolates reacted positively with Ngari (NRI) type-specific ascitic fluid. No group 2 antigen reacted with Germiston virus, but all antigens reacted with antibody to individual Ilesha, Shokwe, Birao, Mboke, and Bozo viruses when tested by the CF test.

One group 3 antigen (20-100) reacted positively with Bagaza (BAG) virus immune serum, but did not react with other antisera to the Flavivirus tested (Saboya, West Nile, yellow fever, Zika, Spondweni, Uganda S, and Usutu). The similarity of 20-100 to BAG was confirmed by PRNT (Table 2). The other group 3 antigen (16-17) did not react with any of the immune sera tested, but did react strongly with Bangui (BGI) virus by the virus neutralization test (Table 3).

DISCUSSION

Ngari virus was the most common isolate (15 isolates), followed by BBK, (three isolates), and BAG and BGI viruses (one isolate each). Ngari (*Bunyavirus*, Bunyaviridae) virus, registered with the American Committee on Arthropod-borne Viruses in 1987, was isolated from six species: *An. pharoensis* (seven), *Cx. antennatus* (two), *Cx. poicillipes* (two), *Cx. tritaeniorhynchus* (two), *Ae. hirsutus* (one), and *An. gambiae* s.l. (one). All isolates represent new mosquito-virus combinations for Senegal. The majority of isolates (11) were collected in the village of Fanaye Diery, while four were collected in Bode. Although both of these locations are in the vicinity of areas where RVF activity was very high in the fall of 1987, we did not isolate RVF virus from mosquitoes.[5]

Minimum field infection rates in Fanaye Diery were as follows: *An. gambiae* s.l. (1:54), *An. pharoensis* (1:89), *Cx. poicillipes* (1:441), *Cx. antennatus* (1:1,062), and *Cx. tritaeniorhynchus* (1:1,976). Ngari virus was first isolated in Senegal in 1979 from male *Ae. simpsoni* mosquitoes reared from field-collected eggs.[17] It has also been previously isolated from *An. gambiae* s.l. in Burkina Faso and the Central African Republic, *An. mascarensis* in Madagascar, and *Ae. argenteopunctatus*, *Ae. vittatus*, and *Ae. neoafricanus* in Senegal.[17]

The numerous isolations in Fanaye Diery and the high minimum field infection rates in mosquitoes in September 1988 suggest that NRI virus was circulating at a high level. The August–September 1988 rainfall was the highest in this region since 1981, and Linthicum and others reported vegetation growth caused by rainfall in the Senegal River at this time to be almost as extensive as in October 1987 when RVF virus was found to be circulating at high levels.[5]

The vertebrates involved in NRI virus amplification are unknown, although the virus has been previously isolated from a sick sheep in southern Mauritania.[18] The numerous isolations of NRI virus from mosquito species that feed on both humans and domestic ungulates (*An. gambiae*, *An. pharoensis*, *Cx. antennatus*, *Cx. poicillipes*, and *Cx. tritaeniorhynchus*)[19] suggest that human and domestic animal involvement in the ecology of NRI virus should be investigated. Prevalence of antibody to NRI in domestic sheep in rural villages in southeastern Mauritania suggests that this virus was circulating in August 1988.[18] Serologic evidence from individuals living near Yonofere, Senegal indicate that humans are infected with NRI virus.[18]

Although NRI virus has been isolated in Kedougou, Senegal, the isolations reported here are the first in northern Senegal. Experimental studies are needed to evaluate the potential for *An. pharoensis*, which accounted for seven of 15 isolates, and other *Anopheles* spp. to serve as vectors of NRI virus. Investigations into the potential

for NRI virus to be transmitted transovarially are warranted based upon the initial isolation of virus from males reared from field-collected eggs and the isolation of the virus from adult female *Aedes* spp., including the isolation reported here from *Ae. hirsutus* collected in Bode.[17]

Babanki (*Alphavirus*, Togaviridae) virus, registered with the American Committee on Arthropod-borne Viruses in 1987, was isolated at three locations: Ndialene (in the delta region), Diongui, and Matam (approximately 400 km upriver). The virus was first isolated in 1969 from *Mansonia africana* in Cameroon and subsequent isolations have been made in *Anopheles*, *Aedes*, *Culex*, *Eretmapodites*, *Coquillettidia*, and *Mansonia* species throughout much of sub-Saharan Africa and Madagascar.[17, 18] This is the first reported isolation of BBK virus from *Cx. tritaeniorhynchus* and *Cx. univittatus* group mosquitoes. Babanki virus has been isolated from humans; however, clinical disease was not described in association with these infections. Calisher and others have reported that BBK virus is closely related to SIN virus.[20] The BBK virus isolates used in this study were also found to be closely related to SIN virus.

Bagaza (*Flavivirus*, Flaviviridae) virus was isolated from *Cx. univitattus* group mosquitoes collected in Matam. The virus was first isolated from *Culex* spp. mosquitoes in the Central African Republic.[18] Subsequently, it was isolated from *Cx. thalassius* in Senegal, *Cx. perfuscus* and *Cx. guiarti* in the Central African Republic, and *Cx. guiarti* and *Cx. ingrami* in Cameroon.[18] However, no information exists concerning natural human or animal infection with this virus.

Bangui (bunyavirus-like, Bunyaviridae) virus was isolated from a single *An. pharoensis* female collected in Ngoui. This virus was first isolated from a human in 1973 and has been associated with human disease.[21] Although Bangui virus resembles members of the Bunyaviridae morphologically,[22] it was not associated with an arthropod until 1985, when it was isolated from *An. nili* in Burkina Faso. The present report is only the second association of Bangui virus with an arthropod.[23] Experimental vector competence studies are needed to determine the relationship between BGI virus and potential mosquito vectors that feed on humans.

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Disclaimer: The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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